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Early development of the neural plate, neural crest and facial region of marsupials

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ABSTRACT

Marsupial mammals have a distinctive reproductive strategy. The young are born after an exceptionally short period of organogenesis and are consequently extremely altricial. Yet because they must be functionally independent in an essentially embryonic condition, the marsupial neonate exhibits a unique suite of adaptations. In particular, certain bones of the facial region, most cranial musculature and a few additional structures are accelerated in their development. In contrast, central nervous system structures, especially the forebrain, are markedly premature at birth, resembling an embryonic d 11 or 12 mouse. This review examines the developmental processes that are modified to produce these evolutionary changes. The focus is on the early development of the neural plate, neural crest and facial region in the marsupial, *Monodelphis domestica*, compared with patterns reported for rodents. Neural crest begins differentiation and migration at the neural plate stage, which results in large accumulations of neural crest in the facial region at an early stage of development. The early accumulation of neural crest provides the material for the accelerated development of oral and facial structures. The first arch region is massive in the early embryo, and the development of the olfactory placode and frontonasal region is advanced relative to the forebrain region. The development of the forebrain is delayed in marsupials relative to the hindbrain or facial region. These observations illustrate how development may be modified to produce evolutionary changes that distinguish taxa. Further, they suggest that development is not necessarily highly conserved, but instead may be quite plastic.

Key words: Heterochrony; mammal; craniofacial development; *Monodelphis*.

INTRODUCTION

Marsupial and placental mammals are distinguished by significant differences in reproductive and life history strategies. Metatherian mammals are born after a short gestation and a particularly short period of organogenesis. The organogenic period ranges from 11 d in some kangaroos, to no more than 2.5 d in many dasyurids (Tyndale-Biscoe & Renfree, 1987; Rose, 1989). All marsupials are highly altricial at birth, with an overall stage of development resembling an 11 or 12 d embryonic mouse, or an approximately 10 wk human embryo. The major portion of morphogenesis and of maternal investment in the young occurs during an extended lactation period. Despite the embryonic state of the neonate, it must be able to independently travel to, identify and attach to the teat. The oral apparatus must be sufficiently developed to suckle, and the major physiological systems (circulatory, respiratory, digestive and excretatory) must be functional. This contrasts with eutherian mammals in which maternal investment is more evenly divided between intrauterine and lactational periods, and most morphogenesis is intrauterine (Renfree, 1983). Even the most altricial eutherian is well developed at birth relative to the marsupial neonate (Eisenberg, 1981). The evolutionary consequences, significance, advantages and disadvantages of these 2 patterns of life history strategy have been discussed in an extensive literature (e.g. Tyndale-Biscoe, 1973;

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Lillegraven, 1975, 1979, 1984; Kirsch, 1977*a*, *b*; Parker, 1977; Russell, 1982; Renfree, 1983, 1995; Hayssen et al. 1985; Lee & Cockburn, 1985; Lillegraven et al. 1987; Thompson, 1987; Tyndale-Biscoe & Renfree, 1987; Cockburn, 1989).

In order for the neonate to function at this overall embryonic state, the morphological configuration must differ from that seen in even the most altricial placental mammal (Esdaile, 1916; Hartman 1919; Hill & Hill, 1955; Müller, 1967, 1972*a*, *b*; Nelson, 1981, 1987; Maier, 1984, 1987, 1993, 1999; Hall & Hughes, 1987; Klima, 1987; Gemmell & Nelson, 1988*a*, *b*, 1992; Hughes & Hall, 1988; Filan, 1991; Clark & Smith, 1993; Gemmell and Selwood, 1994; Smith, 1994; Frigo & Woolley, 1996). There is an overall rostral–caudal gradient in development so that the cranial, cervical and upper thoracic regions are well developed with little development of posterior regions. The forelimb is particularly large, as are the vertebrae in the cervical region. In the head, the chondrocranium is massive, particularly in the nasal region, and the bones around the oral apparatus are developed precociously. Most craniofacial muscles are well developed and the tongue is particularly large. In contrast, the brain is at an embryonic state of development, and the bones around the calvaria have not yet developed (Fig. 1). Furthermore, the jaw joint is uniquely configured. While it superficially resembles the primitive condition, with the joint being formed by the cartilaginous anlagen of the malleus and incus, it also possesses a number of derived features (Filan, 1991; Maier, 1993).

Within marsupials, the variation in degree of development at birth is narrow, and does not match the altricial-precocial spectrum seen in eutherians. However, there is variation between the most and least developed marsupial (Hall & Hughes, 1987; Hughes & Hall, 1988). In phalangers and kangaroos (Diprotodontia), there is generally a single young that weighs between 200 and 800 mg at birth (adult weight between 500 g and 27 kg). In these taxa, young are relatively well developed with prominent external ear and eye primordia, retinal pigmentation, significant differentiation (e.g. digits present) in the hindlimb, and pronounced differentiation of the mandible. The head and neck are morphologically distinct, and mature cartilaginous elements in the head and forelimb (e.g. phalanges) are distinguishable (Hughes & Hall, 1988; Fig. 2*B*). In contrast, the dasyurid neonate is ultra-altricial. In these taxa, the newborn weighs between 5 and 20 mg (adult weight between 8 g and 12 kg), and litter sizes are often as large as 12. These young are remarkable in the rudimentary nature of their development (Fig. 2*A*). At birth, ear and eye primordia are barely visible and the head is virtually all nose and mouth. There is an extreme gradient between the fore- and hindlimbs, so that while the forelimbs are robust, the hindlimbs are barely beyond bud stage. Cartilage is poorly differentiated. For example, in the forelimbs of the newborn Tasmanian devil (*Sarcophilus harrisii*), the metacarpals are present, but the phalanges have not yet differentiated (Hughes & Hall, 1988). Perhaps most unusual is the large mass of undifferentiated mesenchyme that occupies the throat and chest region (Fig. 2*A*). This mass is transient and serves to support passively the head and neck of the highly altricial neonate (Hill & Hill, 1955).

The development of the craniofacial region in marsupials is of particular interest. This region is exceedingly complex functionally, morphologically and developmentally. Many systems, such as the central nervous system, the cranial sense organs, and the skeletal and muscular systems are thought to develop in a highly coordinated manner. Further, in marsupials a number of elements of the craniofacial region must be functional at a time when much of this region is just beginning morphogenesis. It has long been recognised that these functional requirements have contributed to specific heterochronies and that some components of the face and oral region develop early and rapidly in marsupials relative to eutherians.

The patterns of craniofacial development in marsupial and placental mammals have been reported in a series of papers (Smith, 1996, 1997, 2000; Nunn & Smith, 1998). These studies investigated craniofacial development during the late organogenic period, from approximately the evagination of the telencephalon, to the last ossification of all bones in the cranium. The analyses included 4 marsupial species (*Monodelphis domestica*, *Macropus eugenii*, *Perameles nasuta*, *Dasyurus viverrinus*), and 6 placental species (*Felis domestica*, *Manis javanica*, *Mus musculus*, *Sus scrofa*, *Tarsius spectrum*, *Tupaia javanica*). A major conclusion was that, in addition to the patterns discussed above, marsupials were characterised by a delay in the development of major structures in the central nervous system and an advancement of the development of elements in the oral and facial regions.

Specifically, the initial ossification of the dentary, maxillary, premaxillary and exoccipital bones, and the closure of the secondary palate, occurred early in marsupials relative to placentals. Events that were late in marsupials relative to placentals included the evagination of the telencephalon, contact between the olfactory bulb and the olfactory epithelium, layering

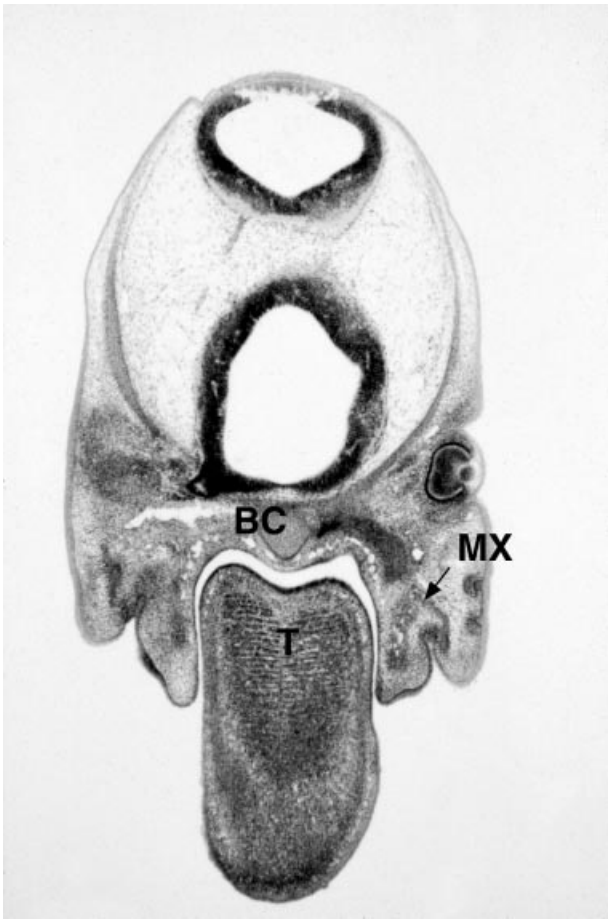

Fig. 1. Transverse paraffin section through the head of a 14 d embryonic *Monodelphis domestica*, about half a day before birth. Note the thin neural epithelium and the early state of differentiation of the eye. These stages are approximately equivalent to a 10.5–11 d embryonic mouse. In contrast, the tongue muscles are differentiated, cartilage is present in the basicranium, and bone is present in the facial region (maxillary, premaxillary and dentary). These structures are equivalent to a 14–15 d embryonic mouse. BC, basicranial cartilage; MX, maxillary bone; T, tongue muscle.

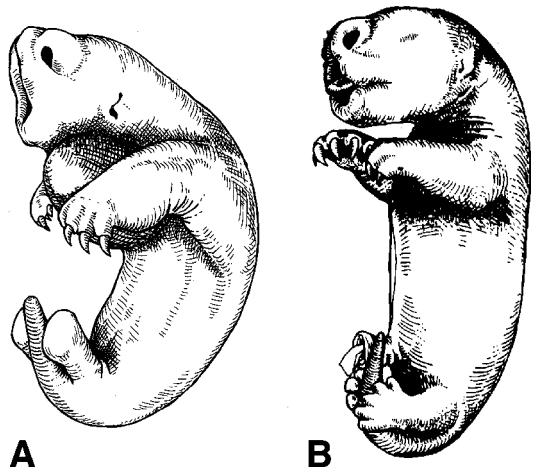

Fig. 2. Embryos of newborn (*A*) *Dasyurus viverrinus* (redrawn from Hill & Hill, 1955), and (*B*) *Trichosurus vulpecula* (redrawn from Klima & Bangma, 1987). These drawings are not to scale. The *D. viverrinus* neonate is 6 mm in length and generally weighs 12.5 mg. The *T.vulpecula* neonate is 15 mm in length and generally weighs 200 mg. Note the extreme difference between fore- and hindlimb development, the existence of a large undifferentiated mass in the thorax and cervical region, and the lack of a recognizable head posterior to the oral-nasal region in *D. viverrinus*.

in the cortex, the differentiation of the thalamus and hypothalamus, filling of the lens vesicle by primary lens cells, and the meeting of the dermal bones over the cranial roof. Therefore craniofacial development in marsupials, when compared with placentals, is distinguished by major shifts in the relative timing of the differentiation of the somatic structures of the head relative to the differentiation of the central nervous system. There are 2 major components of these heterochronies. First, in eutherians the onset of morphogenesis of the CNS begins long before the appearance of any cranial skeletal or muscular tissues. In marsupials, cranial skeletal and muscular tissues begin development early relative to CNS differentiation. Second, in eutherians the events of CNS development examined are completed before most somatic structures begin differentiation, while in marsupials morphogenesis of these same CNS elements extends long into the period of cranial skeletal development.

Thus the competing demands of the very short organogenic period and the necessity for function at a highly altricial state have produced a uniquely configured neonate. The developmental mechanisms underlying the morphological transformation are unknown. Of particular interest is the role of neural crest. Neural crest cells make up much of the connective tissues of the facial region, and appear to be critical in patterning muscular organisation (e.g. Noden, 1983, 1984; Hall, 1999; LeDouarin & Kalcheim, 1999). It might be predicted that the differentiation and migration of neural crest cells might also be advanced in marsupials. Information on neural crest differentiation and migration in marsupials has not been previously published.*

* Katherine P. Watson and J. P. Hill conducted an extensive series of studies on the development of the neural tube in marsupials between 1911 and at least 1946. In the course of this work, they observed the migration of neural crest cells from the neural plate into the facial region. Except for a brief communication of this work to the Anatomical Society of Great Britain and Ireland in 1920, published after Hill's death (Hill & Watson, 1958), this work was never published. I am currently transcribing this work, in which the development of the neural tube and neural crest is described in detail in dasyurids, peramelids, macropodids, with comments on a few other taxa. Despite reports to the contrary (Hall, 1999; Romer, 1972), it appears that Hill and Watson both accepted the contribution of neural crest to the ectomesenchyme of the face by the time of the presentation of the paper in 1920.

In this paper, several aspects of the early development of the craniofacial region in marsupials are discussed. Focus is on early development of the neural tube, differentiation of the neural crest and early formation of the facial region. The central question concerns how development has been modified to produce the unique configuration of the marsupial neonate. This question is important for at least three reasons. First, it is a case study of how developmental mechanisms are modified to produce the kinds of morphological change of interest to evolutionary biologists. Second, the events of cranial development occur at different rates and in different sequences in marsupials and placental mammals. These timing differences mean that elements that are coordinated or apparently integrated in placental mammals may occur at different times, or in difference sequences in marsupials. A comparative study such as this serves as a natural experiment and allows us to probe hypotheses about general developmental mechanisms. Finally, this study serves as a case study of the degree of plasticity or conservation in early development.

EARLY DEVELOPMENT OF THE NEURAL TUBE AND NEURAL CREST IN *MONODELPHIS DOMESTICA*

Early development was studied in the grey short-tailed opossum, *Monodelphis domestica* (Didelphidae). Embryos were staged according to criteria first developed by McCrady (1938) for *Didelphis virginiana*, and later modified by Mate et al. (1994) for *M. domestica*. As the embryos at these stages are flat, very thin, and covered only by transparent membranes, a great deal of early development can be documented by whole embryo examination.

This study begins with stage 22 embryos, which appear approximately 10 d after mating. During this stage somites first appear. This stage is a few hours after the primitive streak stage, stage 18 (Mate et al. 1994). At stage 22, the neural plate is broad and flat and occupies much of the surface of the embryo, with the notochord visible in the midline. At this time the neural plate first begins to exhibit differentiation with the appearance of the preotic and otic sulci. First arch

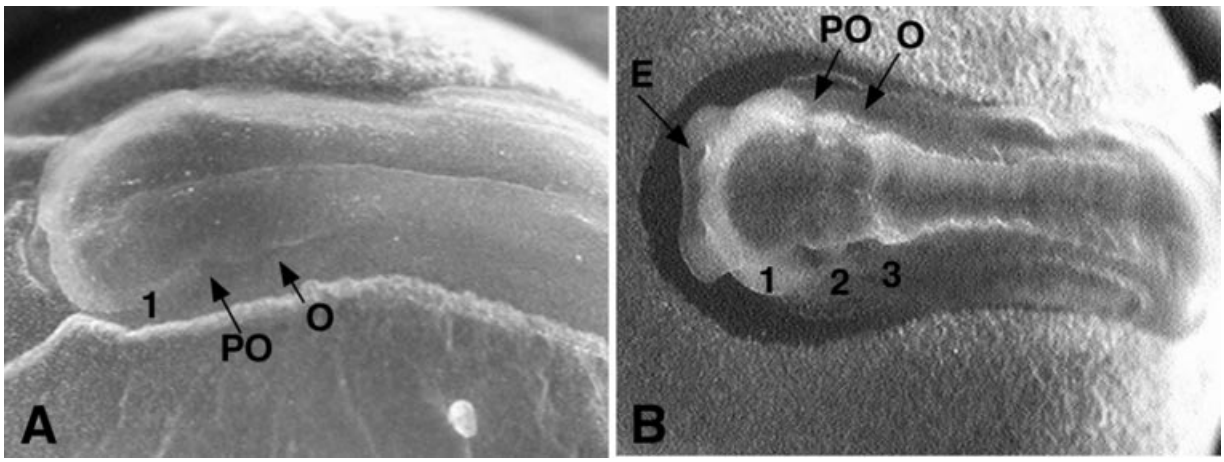

Fig. 3. Embryos of *Monodelphis domestica* at (*A*) stage 22 and (*B*) stage 24. E, optic pit; O, Otic sulcus; PO, preotic sulcus; 1, first arch neural crest; 2, second arch neural crest; 3, region from which third arch neural crest will appear.

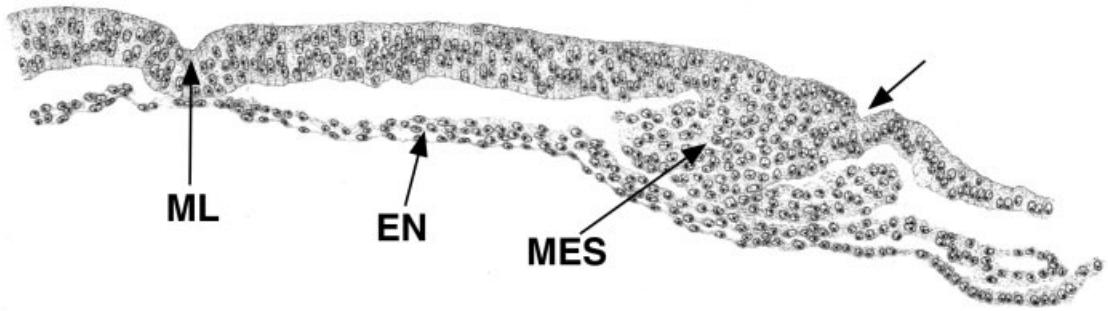

Fig. 4. Unpublished drawing from the Hill and Watson collection at the Hubrecht Comparative Embryology Laboratory; transverse section through the first arch region of a stage 24 *Perameles obesula* embryo (MS 203). The embryo had 4–5 somites. Note the broad mass of mesenchymal cells in continuity with the neural plate. ML, midline of the neural plate; EN, endoderm overlying the yolk sack; MES, first arch mesenchyme of neural crest origin; unlabelled arrow, border of neural plate and ectoderm. At this stage, the mesenchyme represents a sizable accumulation of first arch neural crest.

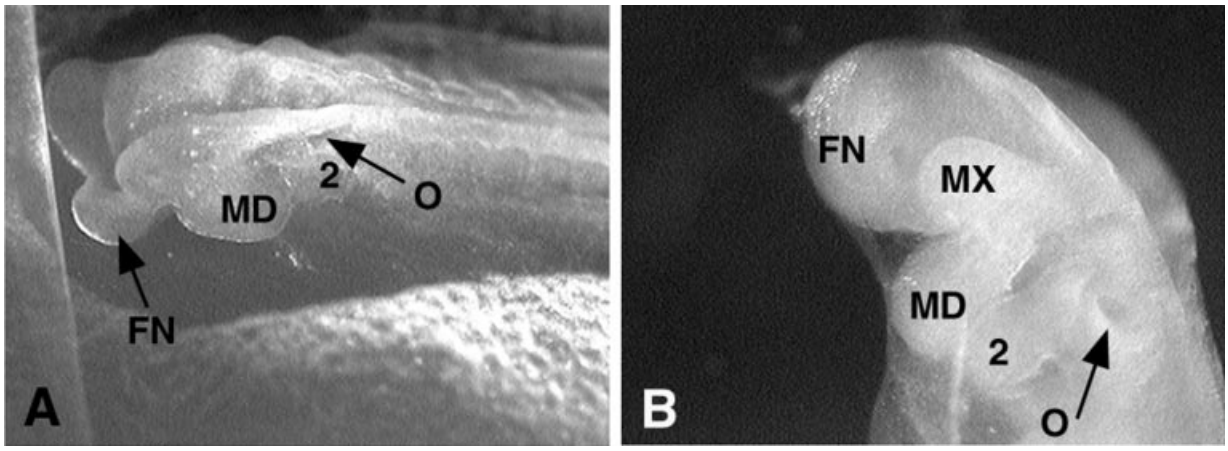

Fig. 5. Photos of embryos of *Monodelphis domestica* at (*A*) stage 25 and (*B*) stage 27. FN, frontonasal process; O, otic vesicle; MD, mandibular process; MX, maxillary process; 2, second arch.

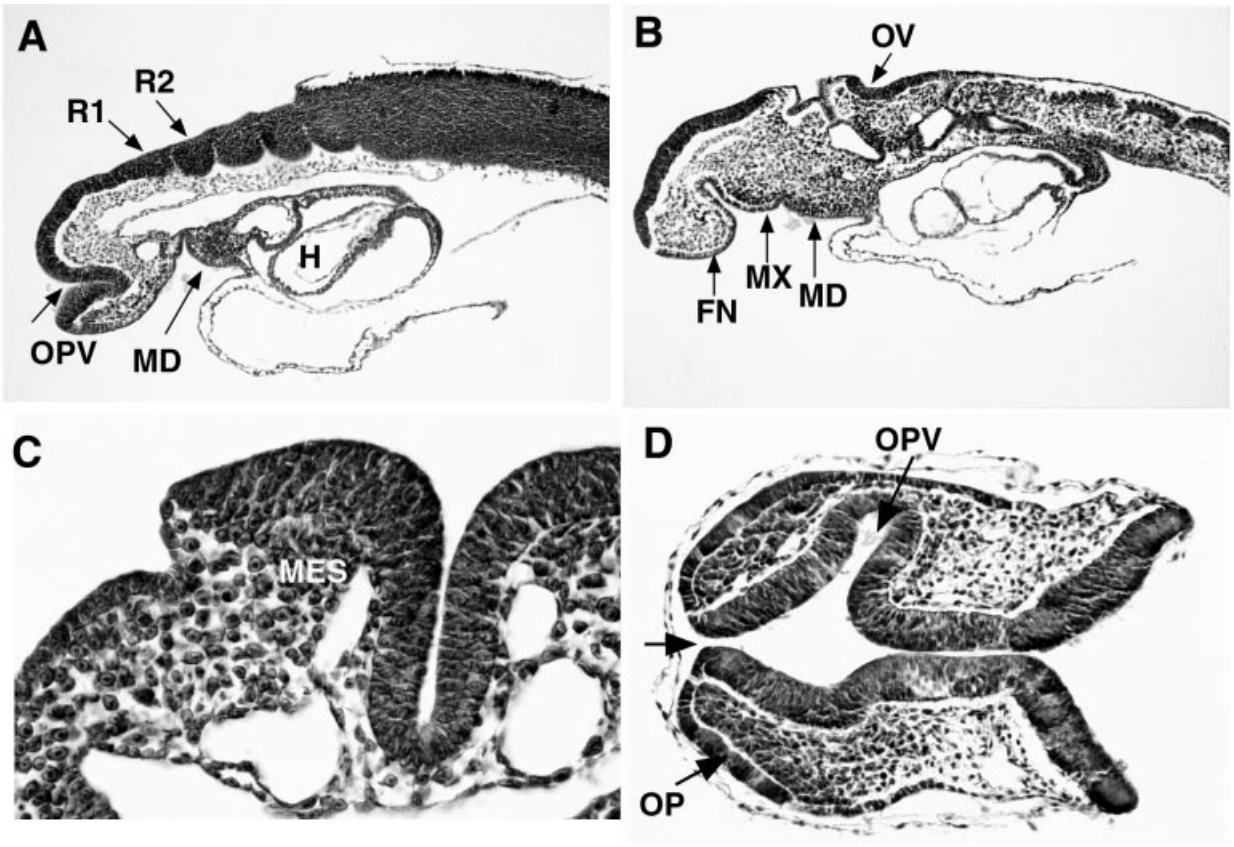

Fig. 6. Photographs of paraffin sections of embryos of *Monodelphis domestica*. Anterior is to the left in *A*, *B*, and *D*. (*A*) Stage 25 embryo, parasagittal section near midline. Note rhombomeres, ventral invagination of optic vesicle, and accumulation of mesenchyme in frontonasal region. (*B*) Stage 25 embryo, parasagittal section, lateral to that in *A*. Note significant accumulation of mesenchyme in frontonasal, maxillary and mandibular processes, pharyngeal pouches 1, 2 and 3. (*C*) Stage 25 embryo cut in cross section, through the region of the first or second rhombomere. Note that mesenchyme is leaving the neuroepithelium from a broad region of the ventral neural plate. (*D*) Stage 27 embryo, cut in coronal section through anterior part of the brain. Note the optic vesicles, the thickened olfactory placode, and the open anterior neuropore. Unlabelled arrow, anterior neuropore; FN, frontonasal processes; H, heart; MD, mandibular processes; MES, neural crest mesenchyme; MX, maxillary processes; OPV, optic vesicles; OV, otic vesicle; OP, olfactory placode; R1&2, first and second rhombomeres. Specimens fixed in Carnoy's fixative, and prepared for paraffin histology using techniques detailed in Smith (1994).

neural crest has begun migration at this stage, before somites appear, from the region just anterior to the preotic sulcus (Fig. 3*A*). At this stage there is no morphological evidence of any subdivision of the neural tube anterior to the preotic sulcus, so it is impossible to define the boundary between the

forebrain, midbrain and hindbrain. It is therefore impossible to identify the region of the brain that gives rise to the first neural crest streams.

The stage 24 embryo (approximately 10.25 d after mating) is characterised by 6–8 somites. At this stage the neural tube begins to initiate closure in the cervical region, but there is no contact of the neural folds, and the heart tubes and vessels first appear. In the head region there is significant accumulation of neural crest in the first arch region and second arch crest is also migrating (Fig. 3*B*). The preotic and otic sulci are well defined. Both optic pits and otic placodes are present, but no significant morphological differentiation of the neural plate anterior to the preotic sulcus is observed. A drawing by Watson and Hill of a section through the first arch region of a stage 24 *Perameles* embryo shows the first arch neural crest arising from the neural plate in a broad area near the junction of the neural plate and the ectoderm (Fig. 4). The illustration shows the general relations of the neural plate, the migrating neural crest and other embryonic structures. At this stage, the neural crest disengages in a broad region from the ventral surface of the very thin neural plate and accumulates in the region between the neural plate and the endoderm overlying the yolk sack.

The stage 25 embryo (approximately 10.5–10.75 d after mating) possesses 12–13 pairs of somites and is characterized by the first contact of the neural folds. The neural tube begins contact in the postotic and cervical regions. At this stage the anterior parts of the hindbrain as well as the mid- and forebrain are still open (Fig. 5*A*), while the optic vesicles have started evagination. Because the optic vesicles begin evagination before there is any expansion of the diencephalons, the initial projection of the optic vesicles is ventral, rather than lateral as seen more commonly (Fig. 6*A*). There is a massive accumulation of mesenchyme in the first arch region (Fig. 6*B*), although neural crest continues to migrate from the region of the first and second rhombomeres (Fig. 6*A*, *C*). The second arch is also well developed and neural crest is migrating in the post otic region. Otic pits are present. In addition, a significant amount of neural crest has accumulated in the frontonasal region (Fig. 6*B*).

Stage 27 embryos (at least 15–18 pairs of somites; 10.5–11 d after mating) are distinguished by a number of features. At this stage both a cephalic flexure in the mesencephalic region (first apparent in stage 26 embryos) and a cervical flexure are present. The amniotic head fold lies in the cervical region. The neural folds are still open anterior to the otic region,

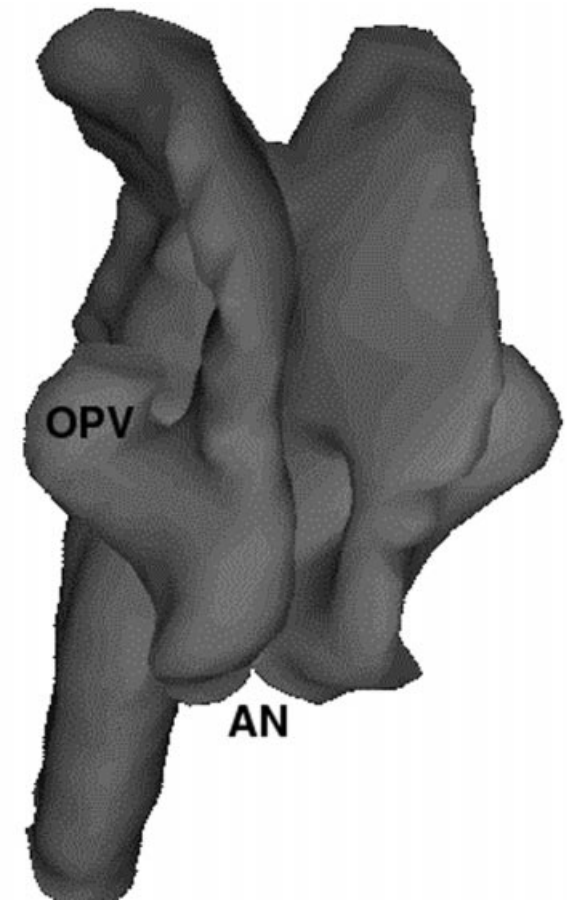

Fig. 7. Three-dimensional reconstruction of stage 27 *Monodelphis domestica* embryo. At this stage, the optic vesicles (OPV) are projecting laterally, but have not yet contacted the ectoderm. The anterior neuropore (AN) is still open, and there is little differentiation of the neural tube anterior to the diencephalon. The reconstruction was made with Surfdriver software (Honolulu, Hawaii), from digital photographs of paraffin sections.

and there is minimal differentiation of the neural tube anterior to the optic vesicles (Fig. 7). The floor of the diencephalon has grown sufficiently so that the optic vesicles now project laterally, but they do not yet contact the epithelium (Fig. 6*D*). Forelimb buds are present as broad, flat, fin-like projections. The maxillary and mandibular processes are distinct (Figs 5*B*, 8), and there is significant accumulation of mesenchyme in the frontonasal region (Fig. 8). Both the second and third arches are distinct. The olfactory placodes are present (Fig. 6*D*). Embryos of this stage were stained with a polyclonal antibody to the distalless protein (Panganiban et al. 1995), which stains *dlx* proteins in mammals. *Dlx* is expressed in migrating neural crest cells (Robinson and Mahon, 1994). A comparison of *Monodelphis* and similarly aged *Mus* embryos highlights the advancement of neural crest relative to the neural tube. A 10 d embryonic mouse shows significantly greater differentiation in the neural tube than a stage 27 *Monodelphis*

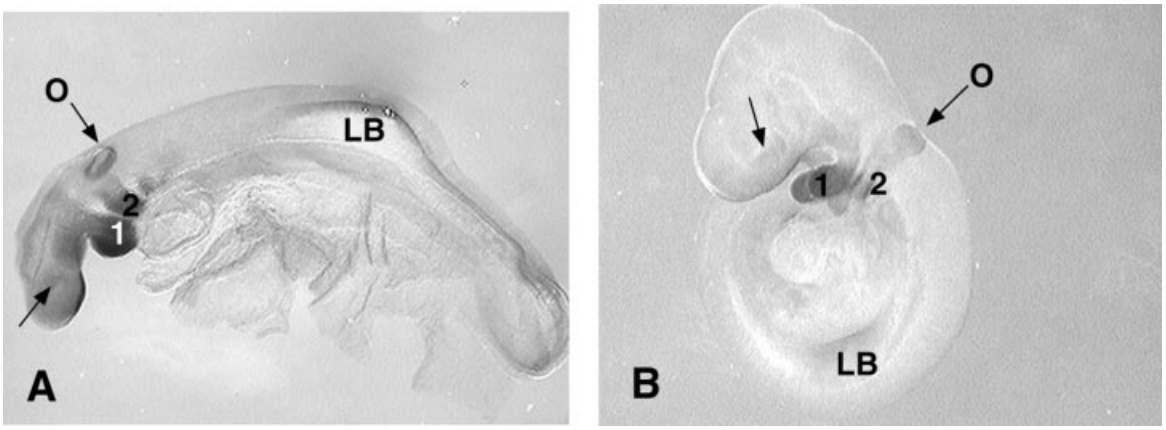

Fig. 8. Embryos of (*A*) stage 27 *Monodelphis domestica*, and (*B*) *Mus musculus* at approximately 9.5 d embryonic, stained with a polyclonal antibody to the *distalless* protein. Photographs are taken at the same magnification. Note that in *Mus* the neural tube is closed and the major regions of the neural tube have differentiated. In *M. domestica*, the neural tube is still open anterior to the otic vesicle, and there is as yet no morphological distinction of mid and forebrain regions. However, the accumulation of neural crest in the first, second and posterior arches is notably advanced in *M. domestica*. There is also very distinct staining in the frontonasal region in *Monodelphis*. Note also the prominent limb bud in *M. domestica*. Unlabelled arrow, position of optic vesicle below the epithelium; O, otic vesicle; 1, first arch; 2, second arch; LB, forelimb bud. Whole mount immunohistochemistry followed techniques published by Hanken et al. (1992, 1997). The polyclonal antibody to distalless protein was provided by Panganiban (Panganiban et al. 1995). The antibody stains the *dlx* protein in mammals, which is expressed in migrating neural crest cells (Robinson & Mahon, 1994; Hanken et al. 1997).

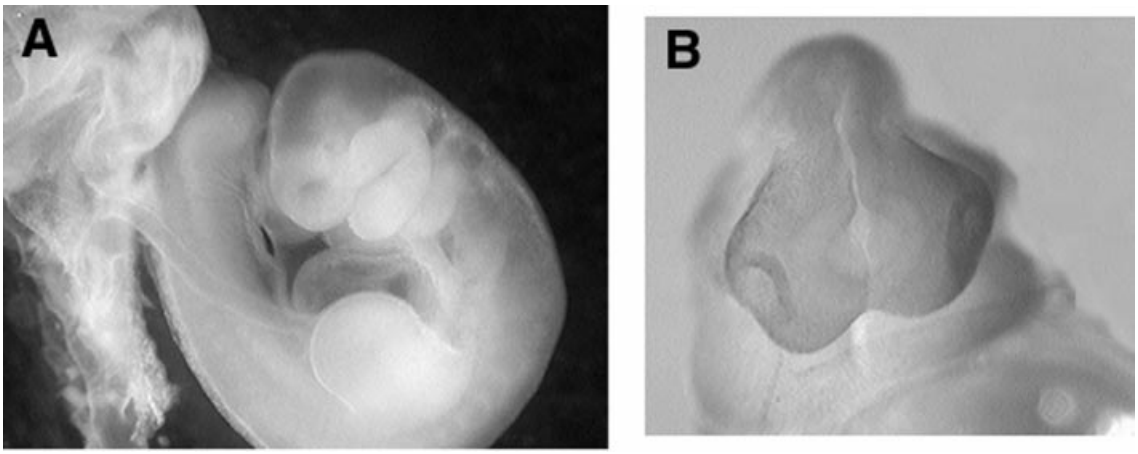

Fig. 9. Embryos of stage 28 *Monodelphis domestica*. Note the massive maxillary and mandibular processes and the large olfactory pits and frontonasal processes.

embryo, but much less accumulation of neural crest in the branchial and facial region.

The anterior neuropore is closed in the stage 28 embryo (11 d after mating), which possess around 25 pairs of somites. This stage is distinguished by the appearance of the primary lumbar flexure, mesonephric tubules and the first appearance of a liver diverticulum (McCrady, 1938). The forelimb buds are rounded. At this stage, the maxillary and mandibular processes are large, and the second arch is well developed. The maxillary process is beginning to fuse with the frontonasal process. The frontonasal processes are particularly large, with massive olfactory pits (Fig. 9). There is still only minor differentiation of the various regions within the prosencephalon.

DISCUSSION

Comparative patterns of neural crest migration

In most vertebrates, neural crest cells migrate only after the neural folds have closed (reviewed in Hall & Horstadius, 1988; Hall, 1999; LeDouarin & Kalcheim, 1999, and references therein). In placental mammals the differentiation of neural crest cells is early relative to these events when compared with birds and other amniotes. In mice and rats neural crest cells begin to differentiate in the first arch region at the 2–3 somite stage before the neural tube has started to close (Nichols, 1981, 1986). First arch crest begins migrating at about the 4 somite stage when the neural tube is just beginning to close. Postotic crest begins

Table. *Major events in the differentiation of the neural tube, sensory organ anlagen, and neural crest in murid rodents and* Monodelphis domestica

Mouse, rat	Number of somites	*Monodelphis*
	0	Preotic sulcus
	0	Otic sulcus
	0	First arch neural crest
Preotic sulcus[1]	~ 1–2	
Otic sulcus[1]	~ 3	
First arch neural crest[2]	~ 4	Second arch neural crest
	~ 4	Optic pits
First contact of neural folds[2]	~ 6–8	
Optic pits[2]	~ 6–8	Post-otic neural crest
Post-otic neural crest[3]	~ 6–8	
Second arch neural crest[3]	~ 8–9	
	~ 12–13	First contact neural folds
	~ 15–18	Olfactory placode
Anterior neuropore closes[4]	~ 20	
Otocyst closes[4]	~ 25–29	Otocyst closes
	~ 25–29	Anterior neuropore closes
Olfactory placode[4]	~ 30–34	

[1] Ruberte et al. (1997); [2] Nichols (1981); [3] Tan & Morriss–Kay (1986); [4] Kaufman & Bard (1999).

migration at the 6–8 somite stage when the neural folds first begin contact, and second arch crest begins migration at the 8–9 somite stage (Nichols, 1981, 1986, 1987; Tan and Morriss-Kay, 1985, 1986; Serbedzija et al. 1992; Morriss-Kay et al. 1993; Osumi-Yamashita et al. 1996). Similar patterns have been reported for macaque monkeys (Peterson et al. 1996). Neural crest migration appears to be different in eutherian mammals from other amniotes in that the postotic crest begins migration before second arch crest (Tan & Morriss-Kay, 1985, 1986; Osumi-Yamashita et al. 1996); in most amniotes, there is a clear rostral–caudal order.

The Table summarises the relative timing of major events in the early development of the neural tube, sensory capsules and neural crest in mice (and rats) and in *Monodelphis*. This comparison reveals four major differences between eutherians and marsupials. First, neural crest migration is early relative to other events in embryonic differentiation. First arch neural crest migration begins before any somites appear. Second, neural tube closure (both the first contact, and the closure of the anterior neuropore) is late relative to other events in *Monodelphis*. Third, the sensory capsules, in particular the otic and olfactory placodes appear early in *Monodelphis*. Finally, in *Monodelphis* there is a clear rostral–caudal gradient in the timing of the migration of the three major streams of neural crest, as has been reported for chick, but not for mouse. If the differentiation of neural crest can be considered the first event in the differentiation of facial skeletal-muscular tissues, this study shows that the basic heterochronies shown to be characteristic of marsupials begin at the earliest cellular events in cranial development.

Developmental plasticity and conservation

The processes that distinguish marsupial and placental mammals begin at the earliest point in the differentiation of tissues of the craniofacial region. They involve fundamental shifts in early patterning events, and comprise changes in a complex series of events. These changes may be traced back to the appearance of the neural plate, where in marsupials large numbers of cells differentiate into migratory neural crest cells rather than neural tissues. Within the neural tube the hindbrain and sensory anlagen differentiate relatively early and the midbrain and particularly forebrain regions are delayed. Within the forebrain, the olfactory bulb is accelerated and cerebral vesicles markedly delayed in onset of development. In addition, there is a localised acceleration of somite differentiation in the cervical and upper thoracic regions and a marked delay in caudal somites. Thus the developmental differences are not merely accelerations of the rates of development of a few features, nor are they due to the establishment of a simple anterior-posterior gradient of acceleration along the body axis. Rather, the changes involve multiple advancements and delays of sets of cells, tissues and organs, within and between regions. The developmental trajectory in

marsupials is highly modified from very early stages in order to produce a specific adaptive configuration of the neonate. This suggests that development, even at its earliest stages, is highly plastic.

The observation of significant early plasticity, even in animals in which the adults are quite similar, is important for understanding the ways in which development and evolution interact. In particular there is much speculation on the conservation of early development (e.g. Wimsatt, 1986) or a conserved phylotypic stage (Slack et al. 1993; Raff, 1996). The changes observed here occur immediately before, during and after the phylotypic stage and include shifts in some of the major patterning events in the body. These observations thus confirm recent studies that suggest that the degree of conservation at a phylotypic stage in vertebrates has been overestimated (e.g. Richardson et al. 1977; Richardson, 1995).

Unresolved questions

This preliminary study suggests that the mechanisms that produce the highly altricial, but functionally independent, neonate in marsupials involve the early differentiation and migration of neural crest. In order to understand the role of neural crest in mediating evolutionary change in this system, many issues must be resolved.

First, we currently do not know when the paraxial mesoderm differentiates. Neural crest and paraxial mesoderm interact to produce the important structures of the facial region. Cranial muscles, which are derived from paraxial mesoderm, are also accelerated in development (Smith, 1994). In the earliest embryos thus far examined, there is no evidence of significant accumulation of mesenchyme in the facial region, aside from the mesenchyme that can be shown to be continuous with the neural epithelium. However, at slightly later stages (e.g. stage 27), when there is sizable accumulation of mesenchyme in the branchial arches, it appears that mesoderm is present. In embryos of this age the *dll* positive cells (presumably neural crest) appear in the periphery of the arches, surrounding a core of mesenchymal cells that do not react with the *dll* antibody. These inner cells are presumably mesodermal cells, as this pattern of a core of mesodermal cells surrounded by neural crest cells has been reported for other amniotes (Trainor & Tam, 1995). The time course of the differentiation of mesoderm and appearance of somitomeres has not yet been documented.

A second question concerns the specificity of fate within the neural crest cells of the first arch. Köntges & Lumsden (1996) demonstrated tremendous specificity of populations of neural crest cells that migrate into the first arch from the first 2 rhombomeres and the midbrain in the chick. The neural crest cells from the midbrain and first rhombomere are found throughout the first arch, while those from the second rhombomere are localized in the posterior margin of the first arch. The cells from the midbrain produce distal elements (e.g. Meckel's cartilage), while the cells from the first two rhombomeres produce proximal elements (e.g. articular, quadrate, squamosal). In *Monodelphis*, the bulk of migration occurs before there is any hint of morphological differentiation of these regions of the brain. It is unknown if these regions exhibit genetic differentiation early relative to morphological differentiation. If genetic differentiation was advanced then even though the midbrain, forebrain and first 2 rhombomeres are not morphologically distinguishable at the time of neural crest migration, they would possess region specific genetic identity. Alternatively, the populations of neural crest may not exhibit the region specific identity seen in the chick. Another question arises from the work of Graham et al. (1996), who have demonstrated the importance of apoptosis in limiting migration of neural crest from rhombomeres 3 and 5. It would be of interest to see if these same processes of neural crest restriction from r3 and r5 are important in marsupials, because at the time of neural crest migration in marsupials the neural tube appears to possess a very limited population of cells.

Finally, it can be predicted that these morphological heterochronies are initiated by heterochronies in gene expression pattern. The patterning of the neural tube and body axis by *hox* and related homeobox genes has received tremendous attention over the past decade. If these genes are important in regional patterning, it can be reasonably hypothesised that the timing of the expression of these regulatory genes will show localised patterns of advancement (in the hindbrain and cervical region), and delay (in the fore- and midbrain and posterior body segments) in marsupials. Likewise, in the forebrain, genes involved in patterning regions such as the olfactory bulb might be expected to occur early relative to other forebrain regions. The comparison of placentals, such as mice, and marsupials such as *Monodelphis*, where very specific morphological heterochronies exist, may be an ideal one to study the relation of morphological and genetic changes, and ultimately the genetic and developmental mechanisms behind evolutionary change.

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